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Bone marrow cells are a rich source of growth factors and cytokines: implications for cell therapy trials after myocardial infarction

Mortimer Korf-Klingebiel[1], Tibor Kempf[1], Thomas Sauer[1], Eva Brinkmann[1], Philipp Fischer[1], Gerd P. Meyer[1], Arnold Ganser[2], Helmut Drexler[1], and Kai C. Wollert[1]*

[1]Department of Cardiology and Angiology, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany; and [2]Department of Haematology, Haemostaseology, Oncology, and Stem Cell Transplantation, Hannover Medical School, 30625 Hannover, Germany

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Aims Results from clinical trials suggest that cardiac function after acute myocardial infarction (AMI) can be enhanced by an intracoronary infusion of autologous unselected nucleated bone marrow cells (BMCs). Release of paracrine factors has been proposed as a mechanism for these therapeutic effects; however, this hypothesis has not been tested in humans.

Methods and results BMCs and peripheral blood leucocytes (PBLs) were obtained from 15 patients with AMI and cultured in serum-free medium to obtain conditioned supernatants (SN). BMC-SN stimulated human coronary artery endothelial cell proliferation, migration, and tube formation, and induced cell sprouting in a mouse aortic ring assay. Moreover, BMC-SN protected rat cardiomyocytes from cell death induced by simulated ischaemia or ischaemia followed by reperfusion. While PBL-SN promoted similar effects on endothelial cells and cardiomyocytes, BMC-SN and PBL-SN in combination promoted synergistic effects. As shown by ProteinChip and GeneChip array analyses (each performed in triplicate), BMCs and PBLs expressed distinct patterns of pro-angiogenic and cytoprotective secreted factors.

Conclusion Our data support the paracrine hypothesis and suggest that characterization of the BMC secretome may lead to an identification of factors with therapeutic potential after AMI.

Keywords Acute myocardial infarction • Cell therapy • Paracrine hypothesis

Introduction

Rapid reperfusion of the infarct-related artery is of critical importance to limit infarct size in patients with acute myocardial infarction (AMI).[1] Unfortunately, myocardial necrosis starts early after coronary occlusion, usually before reperfusion can be achieved. The resulting loss of viable myocardium initiates an inflammatory process that leads to a replacement of the infarcted area with scar tissue, and sets the stage for systolic dysfunction and progressive ventricular remodelling in many patients.[2] Results from randomized-controlled clinical trials suggest that the recovery of left ventricular systolic function in patients after AMI can be enhanced by an infusion of autologous unselected nucleated bone marrow cells (BMCs) into the reperfused coronary artery.[3–5]

While these studies have already triggered additional clinical trials that are currently assessing procedural issues of cell therapy (e.g. cell dosage and timing of cell transfer),[6,7] little is known about the mechanisms of how unselected BMCs may improve systolic function after AMI. Differentiation of haematopoietic stem cells and endothelial precursor cells into cardiomyocytes and endothelial cells has been offered as an explanation,[8,9] but the quantitative importance of cell incorporation has been challenged.[10–13] Animal studies employing specific stem and progenitor cell populations suggest that paracrine signalling may be an additional or alternative mechanism for the therapeutic effects of cell transfer after tissue ischaemia.[13–16]

The significance of these experimental findings with regard to unselected BMCs that are currently applied in clinical trials is

* Corresponding author. Tel: +49 511 532 4055, Fax: +49 511 532 5307, Email: wollert.kai@mh-hannover.de

uncertain. In the present study, we assessed the secretory capacity of unselected BMCs in patients undergoing BMC therapy after AMI. Our data show that BMCs deliver a distinct cocktail of growth factors and cytokines into infarcted myocardium and indicate that characterization of the BMC secretome may lead to the identification of factors with therapeutic potential after AMI.

Methods

Patients and bone marrow cell preparation

The present study is a subinvestigation of the ongoing BOne marrOw transfer to enhance ST-elevation infarct regeneration (BOOST) 2 trial. BOOST 2 is a randomized, placebo-controlled, multicentre trial of intracoronary BMC transfer in patients with a first ST-segment elevation AMI (see http://www.controlled-trials.com/ISRCTN17457407 for the study protocol). Unselected nucleated BMCs were prepared using the same method that was used in the BOOST trial.[3] In brief, bone marrow was aspirated in the late afternoon from the posterior iliac crest during a brief general anaesthesia with midazolam and etomidate, stored at 4°C overnight, and processed the next morning by 4% gelatine-polysuccinate density gradient sedimentation according to current GMP regulations at Cytonet Hannover. From each patient, an aliquot of the final BMC product, corresponding to 20 mL of the initial bone marrow aspirate, and 25 mL of heparinized venous blood were available for research purposes. BMCs and blood samples from 15 patients were used in the present study. Per protocol, BMCs are irradiated prior to intracoronary transfer in one-half of the patients in the BOOST 2 trial. For the present investigation, only non-irradiated cells were used. On average, $2.6 \pm 0.6 \times 10^8$ BMCs were obtained from each patient and taken to the lab in a transfer box at ~4°C. Erythrocytes were depleted from BMCs and blood samples by ammonium chloride lysis. Nucleated BMCs and peripheral blood leucocytes (PBLs) were then snap-frozen for later RNA isolation or cultured for 24 h at 37°C in serum-free DMEM in 6-well plates (5×10^6 cells in 2 mL per well) to obtain conditioned supernatants (SN). SN from patients 1–6 were pooled and used for *in vitro* assays (five males and one female). SN and RNA samples from patients 7–15 were used for ProteinChip and GeneChip arrays (all males). The study was approved by the ethics committee of Hannover Medical School. All patients provided written informed consent.

Angiogenesis assays

Human coronary artery endothelial cells were purchased from Cambrex (Walkersville, MD, USA) and grown in endothelial cell growth medium MCDB131 (Invitrogen, Paisley, UK) supplemented with 10% foetal calf serum (Invitrogen) in 0.1% gelatine-coated T75 flasks. Cells from passages 3 to 7 were used. Prior to their use in functional assays, endothelial cells were cultured overnight in MCDB131 containing 0.5% foetal calf serum. Endothelial cell proliferation was measured by bromodeoxyuridine incorporation in 96-well plates (5×10^3 cells per well). Endothelial cell migration was assessed in Boyden chambers with 8 μm pore size (Costar, Cambridge, MA, USA); 1×10^5 cells were added to the transwell insert. The number of migrated cells on the lower surface of the filter was counted after 24 h. Endothelial cell tube formation was assayed in 24-well plates (3×10^4 cells per well) coated with growth factor-reduced Matrigel (BD Biosciences, Bedford, MA, USA); tube formation was quantified after 6 h by phase contrast microscopy.[17] Vascular cell sprouting was assessed in an aortic ring assay, as described.[18] In brief, mouse aortic rings were embedded in growth factor-reduced Matrigel in 24-well plates, and cultured for up to 2 weeks in MCDB131 with 1% foetal calf serum. Cellular outgrowth was assessed by phase contrast microscopy and expressed as maximum sprout length.[18] Endothelial cells and aortic rings were stimulated with BMC-SN and/or PBL-SN at different concentrations. Negative controls were stimulated with unconditioned serum-free DMEM; positive controls were stimulated with vascular endothelial growth factor (VEGF) (10 ng/mL) or fibroblast growth factor (FGF) 2 (100 ng/mL).

Cardiomyocyte cell death assays

Ventricular cardiomyocytes were isolated from 1 to 3 day old Sprague–Dawley rats by Percoll density gradient centrifugation.[19] Cells were plated in gelatin-coated culture dishes in DMEM/medium 199 (4:1), supplemented with 10% horse serum, 5% foetal calf serum, glutamine, and antibiotics. The next morning, cells were switched to DMEM/medium 199 supplemented only with glutamine and antibiotics (maintenance medium). Cells were exposed to simulated ischaemia (4 h), or simulated ischaemia (3 h) followed by reperfusion (1 h), as described.[20,21] In brief, cells were switched from maintenance medium to a buffer containing (in mmol/L) 137 NaCl, 12 KCl, 0.5 $MgCl_2$, 0.9 $CaCl_2$, 4 HEPES, 10 2-deoxy-glucose, and 20 sodium lactate (pH 6.2), and were incubated at 37°C in a hypoxia chamber (Modular Incubator Chamber-101, Billups-Rothenberg) flushed with 5% CO_2 and 95% N_2 (simulated ischaemia). Control cells were cultured in a buffer containing (in mmol/L) 137 NaCl, 3.8 KCl, 0.5 $MgCl_2$, 0.9 $CaCl_2$, 4 HEPES, 10 glucose, and 20 pyruvate (pH 7.4), and incubated at 37°C in an atmosphere containing 5% CO_2 and 95% room air. After various time intervals, cells were switched back to maintenance medium and kept in 5% CO_2 and 95% room air at 37°C (simulated reperfusion). Cardiomyocyte necrosis was assessed by ethidium homodimer III (EthIII) staining and Hoechst 33258 counter-staining. EthIII-positive cells were quantified by fluorescence microscopy.[22] Apoptotic cell death was assessed by *in situ* TdT-mediated dUTP nick end-labelling (TUNEL) using the ApopTag fluorescein apoptosis detection kit from Millipore (Billerica, MA, USA) and DAPI counter-staining. The number of TUNEL-positive nuclei displaying condensed nuclear chromatin was determined by fluorescence microscopy.[21] As an additional measure of apoptotic cell death, we assessed the formation of histone-associated DNA fragments by the Cell Death Detection ELISA from Roche (Basel, Switzerland).[21] Cardiomyocytes were stimulated with BMC-SN and/or PBL-SN at different concentrations. Negative controls were stimulated with unconditioned serum-free DMEM; positive controls were stimulated with growth differentiation factor 15 (GDF15) (20 ng/mL).

ProteinChip array

Human Cytokine ProteinChip Array C Series 2000 (Ray Biotech, Norcross, GA, USA) membranes targeting 174 secreted factors were incubated with 1 mL of BMC-SN or PBL-SN at room temperature for 2 h, washed, and then sequentially incubated with biotinylated antibodies, and horseradish peroxidase-conjugated strepavidin and detection solution according to the manufacturer's instructions.

GeneChip array

Total RNA was isolated from BMCs and PBLs using the RNeasy Kit from Qiagen (Hilden, Germany). RNA was biotinylated and hybridized to Affymetrix Human Genome U133 plus 2.0 GeneChip Arrays which allow analysis of 54 675 transcripts. All data were exported into GeneSpring 7.2 (Silicon Genetics, Foster City, CA, USA). Genes that were differentially expressed at least two-fold were further analysed using the GeneOntology freeware (http://www.geneontology.org). Genes

were filtered to include genes annotated with the term 'extracellular' and to exclude genes annotated with the terms 'integral to membrane', 'nucleus', 'cytoplasm', or 'intracellular'.

Quantitative PCR

After reverse transcription (Superscript II, Invitrogen), QPCR was performed using gene-specific oligonucleotide primers from Biomol (Hamburg, Germany), and the Brilliant SideStep SYBR Green QPCR Master Mix and Mx4000 Multiplex QPCR System from Stratagene (Amsterdam, The Netherlands). Expression was normalized to GAPDH mRNA expression levels.

Enzyme-linked immunosorbent assay

The concentrations of bone morphogenetic protein 2 (BMP2), dickkopf homolog 1 (DKK1), FGF9, and VEGF were measured by ELISA in BMC-SN and PBL-SN using ELISA kits from R&D Systems (Wiesbaden, Germany) and a plate reader from BioTek (Bad Friedrichshall, Germany).

Statistical analyses

Data are presented as mean ± SEM. Differences between groups were analysed by one-way ANOVA followed by Student–Newman–Keuls test. A two-tailed *P*-value <0.05 was considered to indicate statistical significance.

Results

Patient population

Patients (14 males and one female) had a mean age of 53 ± 3 years. Thirteen patients presented with an anterior or lateral AMI and two patients with an inferior AMI. Coronary angiography, angioplasty, and stent implantation were performed 5.9 ± 0.8 h after symptom onset. The mean time from coronary intervention to BMC harvest was 6.5 ± 0.4 days. Patient characteristics are shown in *Table 1*.

Table 1 Patient characteristics

Demographics	$n = 15$
Age (years)	53 ± 3
Male gender, *n*	14
Myocardial infarct	
Delay time (h)	5.9 ± 0.8
Infarct-related artery	
LAD	$n = 12$
RCA	$n = 2$
LCX	$n = 1$
Maximum CK (U/L)	3762 ± 444
Time from PCI to BMC harvest (days)	6.5 ± 0.4
Cardiovascular risk factors	
Hypertension, *n*	5
Hypercholesterolemia, *n*	6
Diabetes, *n*	3
Current smoking, *n*	6
Medication at the time of BMC harvest	
Aspirin and clopidogrel, *n*	15
β-Blocker, *n*	15
ACE inhibitor/AT_1 blocker, *n*	15
Statin, *n*	15

Data are presented as numbers, *n* or mean (± SEM). Delay time refers to the time from symptom onset to percutaneous coronary intervention (PCI). LAD, left anterior descending coronary artery; LCX, left circumflex coronary artery; RCA, right coronary artery; CK, creatine kinase; BMC, bone marrow cell.

Conditioned bone marrow cell supernatants promote pro-angiogenic effects

BMC-SN stimulated human coronary artery endothelial cell proliferation (*Figure 1A*), migration (*Figure 1B*), and tube formation (*Figure 1C*) in a dose-dependent manner. Moreover, BMC-SN stimulated cell sprouting from cultured aortic rings (*Figure 1D*). The pro-angiogenic effects achieved with BMC-SN in these assays were comparable with the effects observed after stimulation with optimum concentrations of the pro-angiogenic factors VEGF or FGF2 (VEGF and FGF2 dose-response curves were obtained in pilot experiments). PBL-SN promoted similar dose-dependent effects on endothelial cell proliferation, migration, and tube formation, and aortic ring cell sprouting (*Figure 1A–D*). Notably, BMC-SN and PBL-SN in combination promoted synergistic effects as shown in the cell migration and aortic ring assays (*Figure 1B* and *D*) (in panel B, BMC-SN combined with PBL-SN, each at a dilution of 0.5×10^{-1}, promoted greater effects when compared with BMC-SN or PBL-SN used separately at a dilution of 1×10^{-1}; in panel D, BMC-SN combined with PBL-SN, each at a dilution of 1.5×10^{-2}, promoted greater effects when compared with BMC-SN or PBL-SN applied separately at a dilution of 3×10^{-2}).

Conditioned bone marrow cell supernatants protect cardiomyocytes from cell death

Four hours of simulated ischaemia induced cardiomyocyte necrosis as shown by EthIII staining (*Figure 2A* and *B*). Consistent with a previous study from our group,[21] the same treatment did not increase apoptotic cell death, as indicated by TUNEL/Hoechst staining and histone ELISA (data not shown). Simulated ischaemia for 3 h followed by reperfusion for 1 h, however, strongly induced cardiomyocyte apoptosis (*Figure 2C–E*). BMC-SN dose-dependently protected cardiomyocytes from necrosis induced by simulated ischaemia, and from apoptosis induced by simulated ischaemia followed by reperfusion (*Figure 2A–E*). The effects were somewhat less pronounced when compared with an optimum dose of GDF15 which was used as a positive control.[21] While PBL-SN promoted similar dose-dependent cytoprotective effects in cultured cardiomyocytes (*Figure 2A*, *C*, and *E*), BMC-SN and PBL-SN in combination promoted synergistic effects and provided enhanced protection against cardiomyocyte necrosis and apoptosis (*Figure 2A* and *C*) (BMC-SN combined with PBL-SN, each at a dilution of 0.5×10^{-2}, promoted greater effects when compared with BMC-SN or PBL-SN used separately at a dilution of 1×10^{-2}).

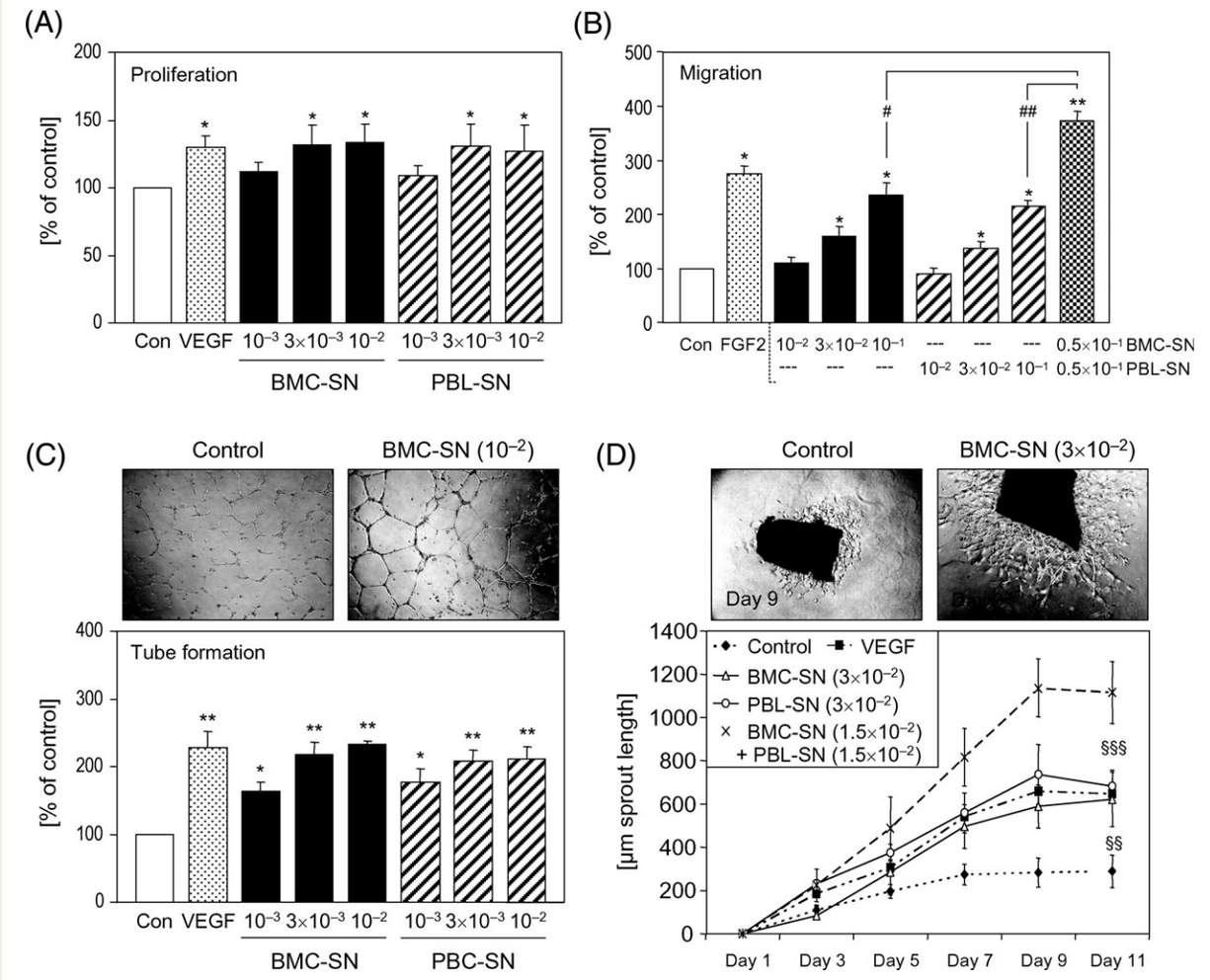

Figure 1 Pro-angiogenic effects of bone marrow cell supernatants. Effects of conditioned bone marrow cell supernatants (BMC-SN) and conditioned peripheral blood leucocyte supernatants (PBL-SN) on human coronary artery endothelial cell proliferation (*A*), migration (*B*), and tube formation (*C*), and on cell sprouting in the mouse aortic ring assay (*D*). BMC-SN and PBL-SN dilution factors are indicated. VEGF (10 ng/mL), and FGF2 (100 ng/mL) were used as positive controls. Representative phase contrast microscopy images are shown in (C and D). Data are from $n = 3–6$ independent experiments; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control (Con); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. BMC-SN or PBL-SN alone; $^{\S\S}P < 0.01$ BMC-SN, PBL-SN, and VEGF vs. control; $^{\S\S\S}P < 0.001$ (BMC-SN+PBL-SN) vs. BMC-SN, PBL-SN, and VEGF.

Bone marrow cells and peripheral blood leucocytes express quantitatively distinct sets of secreted factors

Postulating that secreted factors contribute to the therapeutic effects of BMCs after intracoronary transfer, we reasoned that factors that may be important in this regard should be expressed more strongly in BMCs when compared with PBLs that are constantly passing through the coronary vascular bed. We therefore decided to search for and focus on secreted factors that are differentially expressed by BMCs vs. PBLs.

ProteinChip arrays were used to identify factors secreted from BMCs and PBLs in patients after AMI. Secreted factors that were differentially expressed at least two-fold in BMC-SN vs. PBL-SN in three independent array analyses (each investigating pooled SN from three patients) are shown in Supplementary material online, *Table S1*. Out of 174 secreted factors represented on the ProteinChip array, 25 factors were present in higher concentrations in BMC-SN, and 10 factors were found in higher concentrations in PBL-SN. Factors secreted more strongly from BMCs included angiogenin and VEGF, hepatocyte growth factor (HGF), insulin-like growth factor 1 (IGF1), interleukin (IL) 10, chemokine (C-C motif) ligand 2 (CCL2), CCL23, and CCL24, chemokine (C-X-C motif) ligand 6 (CXCL6), CXCL12, and CXCL13, and FGF9. Factors secreted more strongly from PBLs included placental growth factor (PlGF) and IL11.

Affymetrix GeneChip arrays were used to screen for secreted factors expressed by BMCs and PBLs on a genome-wide scale.

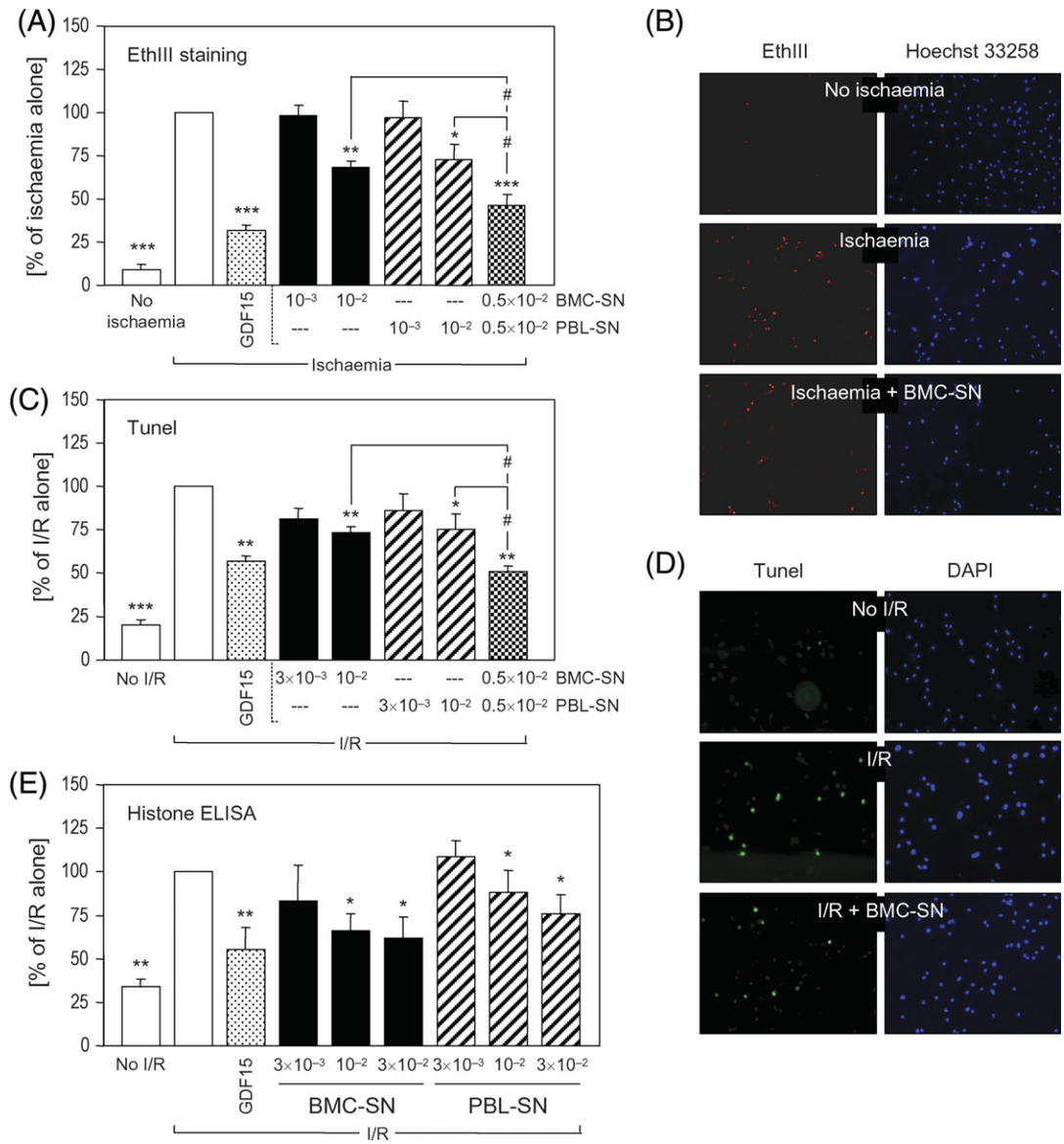

Figure 2 Cytoprotective effects of bone marrow cell supernatants. Effects of conditioned bone marrow cell supernatants (BMC-SN) and conditioned peripheral blood leucocyte supernatants (PBL-SN) on rat ventricular cardiomyocyte death. Cells were exposed to 4 h of simulated ischaemia (*A* and *B*) or to 3 h of simulated ischaemia followed by 1 h of reperfusion (I/R, panels *C*–*E*). BMC-SN and PBL-SN dilution factors are indicated [10^{-2} in (B) and (D)]. Necrotic cell death was assessed by EthIII staining (A and B). Apoptotic cell death was assessed by TUNEL (C and D) and by histone ELISA (E). Representative EthIII/Hoechst 33258 and TUNEL/DAPI stainings are shown (B and D). Growth differentiation factor 15 (GDF15, 20 ng/mL) was used as a positive control. Data are from $n = 3$–6 independent experiments; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. ischaemia or I/R alone; $^{\#}P < 0.05$ vs. BMC-SN or PBL-SN alone.

Factors that were differentially expressed on the mRNA level at least two-fold in three independent array analyses (each investigating pooled RNA from 3 patients) are listed in Supplementary material online, *Table S2*. Overall, 125 secreted factors were expressed more strongly by BMCs, whereas 70 secreted factors were expressed more strongly by PBLs. Eight BMC-overexpressed factors were chosen for QPCR investigation. Stronger expression in BMCs when compared with PBLs was confirmed for all eight factors (*Figure 3A*), thus supporting the validity of the GeneChip array data. Protein concentrations of four of these factors were also measured by ELISA in BMC-SN and PBL-SN (BMP2, DKK1, FGF9, VEGF). All of these factors were detectable in significantly higher concentrations in BMC-SN when compared with PBL-SN (*Figure 3B*).

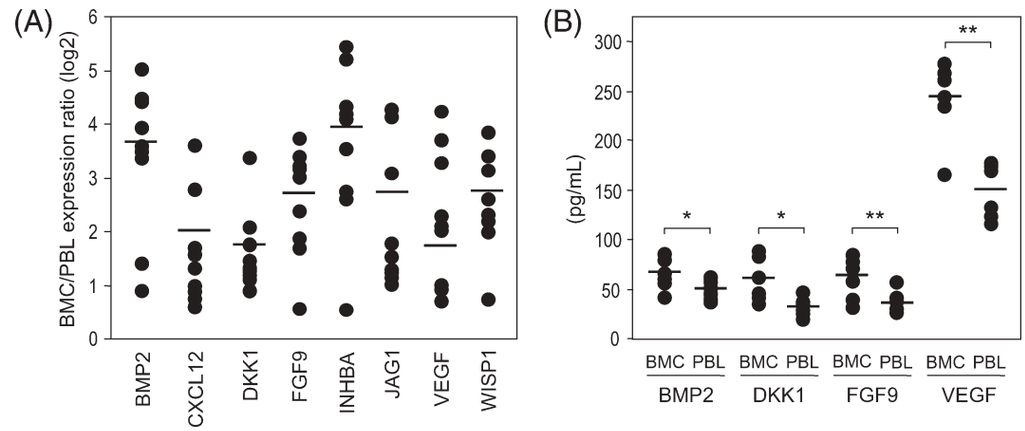

Figure 3 Validation of bone marrow cell-overexpressed factors. (*A*) Eight secreted factors that were preferentially expressed by bone marrow cells (BMCs) when compared with peripheral blood leucocytes (PBLs) according to the GeneChip arrays were chosen for QPCR validation. Expression levels in BMCs and PBLs were measured in six patients previously included in the GeneChip analyses and three additional patients. Data are presented as log2 transformed BMC/PBL expression ratios. (*B*) Concentrations of four secreted factors were determined by ELISA in BMC- and PBL-conditioned supernatants in three patients that were included in the GeneChip analyses and three additional patients. Means are indicated by horizontal bars. $*P < 0.05$, $**P < 0.01$.

Discussion

The mechanisms whereby unselected nucleated BMCs improve left ventricular systolic function in patients with AMI are poorly understood and difficult to explore in a clinical setting. The present study shows that BMCs, which are currently used in cell therapy trials in patients after AMI, secrete pro-angiogenic and cytoprotective growth factors and cytokines, and can promote angiogenesis and cardiomyocyte survival via paracrine effects.

Experimental studies indicate that enhanced angiogenesis and cytoprotection may both contribute to the effects of stem and progenitor cell therapy after myocardial infarction. Increased angiogenesis has been postulated to improve infarct healing and energy metabolism in the infarct border zone.[23–26] Cytoprotective effects may salvage cardiomyocytes at risk and lead to a reduction in infarct size.[15,27,28] In one clinical trial, improvements in left ventricular systolic function in AMI patients undergoing intracoronary BMC transfer have been found to be associated with improvements in microvascular function and tissue perfusion in the infarcted area.[29] Other clinical data indicate that BMC transfer, when applied early after coronary reperfusion, may lead to a reduction in myocardial infarct size.[30] Our data indicate that such effects could be related to paracrine signalling between transplanted BMCs and resident endothelial cells and cardiomyocytes. In line with this conclusion, conditioned BMC-SN obtained from patients with advanced coronary artery disease and refractory angina have been found to contain VEGF and CCL2 and to stimulate the proliferation of human umbilical vein endothelial cells *in vitro*.[31,32]

BMC-mediated paracrine effects suggest that a systematic analysis of the BMC secretome may lead to the identification of (new) cardioactive factors. Considering (i) that PBLs are constantly passing through the coronary vascular bed of a reperfused infarct, (ii) that PBLs have previously been found to express several cytokines and growth factors,[33] and (iii) that conditioned PBL-SN promoted similar pro-angiogenic and cytoprotective effects in our study, we reasoned that cytokines and growth factors that are more strongly expressed by BMCs when compared with PBLs may be especially important for any therapeutic effects of BMC transfer. As shown by ProteinChip and GeneChip array analyses, BMCs and PBLs expressed quantitatively distinct patterns of pro-angiogenic (e.g. angiogenin, VEGF, and PlGF)[34–36] and cytoprotective paracrine factors (e.g. IGF1 and IL11).[37,38] Angiogenin, VEGF, and IGF1 were secreted more strongly from BMCs, whereas PlGF and IL11 were secreted more strongly from PBLs. Differential expression of secreted factors provides a rationale why BMC-SN and PBL-SN promoted synergistic, and not only additive effects in our assays.

While our study was focused on pro-angiogenic and cytoprotective effects of BMC secreted factors, additional downstream effects are possible. IGF1 and HGF, for example, have been shown to act on cardiac resident stem and progenitor cells.[39] IGF1 also provides survival signals to transplanted cells themselves,[40] and modulates the inflammatory response.[37,41] CXCL12 (stromal cell-derived factor 1) can promote tissue neovascularization by recruiting circulating progenitor cells.[42] BMP2 and the canonical Wnt signalling inhibitor DKK1 may enhance cardiomyocyte survival and wound healing after myocardial infarction.[43,44] As shown in our study, all of these factors were preferentially secreted from BMCs when compared with PBLs; in addition, several chemokines and the anti-inflammatory cytokine IL10 were secreted more strongly from BMCs, suggesting that BMCs may influence the inflammatory response after AMI.

Some limitations of the present study need to be acknowledged. First, little is known about which BMC subpopulations are retained in the infarcted area after intracoronary delivery in patients.

We, therefore, focused on the unselected BMC preparation that has been used in the BOOST trial and that is currently used in BOOST 2. We have previously shown that $CD34^+$ cells are retained more efficiently when compared with unselected BMCs after intracoronary infusion in patients with AMI.[45] Accordingly, patterns of secreted factors expressed by cells that undergo intracoronary infusion and cells that are retained in the infarcted area may be somewhat different. Second, it is not known which secreted factors are produced by BMCs in the heart after intracoronary transfer. As recently observed after endothelial progenitor cell transplantation in a mouse model of AMI, transplanted cells may stimulate resident cells to produce additional secreted factors.[28] Third, secretome analyses were performed only in male patients, and we did not assess potential gender differences in the secretory capacity of BMCs and PBLs.

In conclusion, our study supports the paracrine hypothesis and indicates that BMCs deliver a distinct panel of secreted factors into the infarcted myocardium. Identification of BMC secreted cardioactive factors may ultimately lead to new therapeutic strategies to positively influence infarct healing and cardiac remodelling.

Supplementary material

Supplementary Material is available at *European Heart Journal* Online.

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